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Post-transplantation Cyclophosphamide and Sirolimus after Haploidentical Hematopoietic Stem Cell Transplantation Using a Treosulfan-based Myeloablative Conditioning and Peripheral Blood Stem Cells

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A B S T R A C T

Haploidentical hematopoietic stem cell transplantation (HSCT) performed using bone marrow (BM) grafts and post-transplantation cyclophosphamide (PTCy) has gained much interest for the excellent toxicity profile after both reduced-intensity and myeloablative conditioning. We investigated, in a cohort of 40 high-risk hematological patients, the feasibility of peripheral blood stem cells grafts after a treosulfan-melphalan myeloablative conditioning, followed by a PTCy and sirolimus-based graft-versus-host disease (GVHD) prophylaxis (Sir-PTCy). Donor engraftment occurred in all patients, with full donor chimerism achieved by day 30. Post-HSCT recovery of lymphocyte subsets was broad and fast, with a median time to CD4 $>$ 200/μL of 41 days. Cumulative incidences of grade II to IV and III-IV acute GVHD were 15% and 7.5%, respectively, and were associated with a significant early increase in circulating regulatory T cells at day 15 after HSCT, with values $<$ 5% being predictive of subsequent GVHD occurrence. The 1-year cumulative incidence of chronic GVHD was 20%. Nonrelapse mortality (NRM) at 100 days and 1 year were 12% and 17%, respectively. With a median follow-up for living patients of 15 months, the estimated 1-year overall and disease-free survival (DFS) was 56% and 48%, respectively. Outcomes were more favorable in patients who underwent transplantation in complete remission (1-year DFS 71%) versus patients who underwent transplantation with active disease (DFS, 34%; $P = .01$). Overall, myeloablative haploidentical HSCT with peripheral blood stem cells (PBSC) and Sir-PTCy is a feasible treatment option: the low rates of GVHD and NRM as well as the favorable immune reconstitution profile pave the way for a prospective comparative trial comparing BM and PBSC in this specific transplantation setting.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative therapy for many malignant and nonmalignant hematological disorders [1]. Although HLA-identical siblings or HLA-matched unrelated donors (MUD) are considered the ideal sources of hematopoietic stem cells, many patients lack timely access to a suitable

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matched donor, especially in the context of highly aggressive disease. A promising alternative stem cell source is the HLA-haploidentical mismatched family donor who is readily available for nearly all patients [2].

Historically, transplantation across the major HLA barrier has been hampered by alloreactivity, manifested in the early attempts as a high incidence of severe graft-versus-host disease (GVHD) and graft rejection [3]. Subsequent strategies focusing on stringent ex vivo T cell depletion of the graft, often coupled with intensive preparative regimens, proved effective in preventing GVHD but at the expense of delayed immune reconstitution and high rate of nonrelapse mortality (NRM) [4,5]. In this setting, cell-based strategies to boost post-transplantation immune recovery could efficiently abate infectious mortality [6,7] but are, at present, difficult to perform outside highly specialized centers.

In recent years, several groups have devised successful approaches to perform T cell–replete transplantation, even in the full haplotype-mismatched setting. Among other approaches [8-10], that which has gained the most interest has been the use of high-dose post-transplantation cyclophosphamide (PTCy) as in vivo T cell–allo-depleting agent [11,12]. This approach has demonstrated promising results, including acceptable rates of NRM and severe GVHD in single- and multi-institution phase II trials and achieving outcomes equivalent to those of HSCT performed using HLA-identical donors or MUD [13].

Haploidentical HSCT with PTCy (haplo-PTCy), as originally devised by Luznik et al., relied on nonmyeloablative conditioning and bone marrow (BM) as graft source [14]. Although associated with low rates of GVHD and NRM, such transplantation strategy was somehow limited by relatively high relapse rates. Recently, different groups have reported encouraging outcomes using peripheral blood stem cell (PBSC) grafts [15-17], myeloablative conditioning regimens [18], or a combination of both [19]. Thus, although different conditioning regimens and stem cell sources have been tested in the context of haplo-PTCy, reported GVHD prophylaxis regimens have relied only on calcineurin inhibitors (CNIs) to date. Current evidence suggests that CNIs negatively affect regulatory T cells (Treg) [20], which are needed to establish appropriate peripheral tolerance after transplantation and to prevent GVHD [21]. In contrast to CNIs, sirolimus is able to foster post-transplantation Treg recovery [10,22], a property also described for PTCy in the absence of CNIs [23,24]. Besides promoting Treg expansion [25], sirolimus may have several additional advantages over other immunosuppressive agents, thanks to its pleiotropic effects, comprising inhibition of antigen presentation and dendritic cell maturation [26-28], antifibrotic activity [29,30], antiviral properties [31], and, most importantly, direct antitumor activity [32-35]. Therefore, in the present report, we investigated whether the combination of a PBSC graft, intensified myeloablative conditioning, and postgraft sirolimus instead of tacrolimus could help increase the feasibility, and possibly also the therapeutic index, of haplo-PTCy.

PATIENTS AND METHODS

Patients

A cohort of 40 consecutive patients with high-risk hematological malignancies at the San Raffaele Haematology and Bone Marrow Transplantation unit received a myeloablative regimen with sirolimus-PTCy (Sir-PTCy) between November 2012 and July 2014. All patients were treated according to current institutional programs upon written informed consent for transplantation procedures and biological sampling. Patients were considered to be in complete remission if disease activity could not be documented by BM evaluation or imaging, depending on the underlying disease. All other patients not falling within this definition were categorized as having active disease. Additionally, patients were stratified by status at the time of transplantation according to the revised disease risk index (DRI) defined by Armand et al. [36]. Because the DRI has been validated only for patients at first transplantation, we considered patients with a previous allogeneic HSCT in a distinct category. Comorbidities at time of transplantation were evaluated according to the comorbidity-age index [37], and predicted natural killer (NK) cell alloreactivity according to the model developed by Ruggeri et al. [38].

HLA Matching

Donors were patients' relatives, defined as biological parents, siblings, children, or cousins. HLA compatibility among donor-recipient pairs was assessed by 10 loci molecular typing (HLA-A, -B, -C, -DRB1, and -DQB1) at the allelic level. Whenever possible, haplotype segregation was determined based on the typing of first-degree relatives.

Conditioning Regimen and GVHD Prophylaxis

Myeloablative conditioning consisted of treosulfan (14 g/m^2/day) on days −6 to −4, fludarabine (30 mg/m^2/day) on days −6 to −2, and melphalan (70 mg/m^2/day) on days −2 and −1, followed by T cell–replete granulocyte colony–stimulating factor (G-CSF)–mobilized PBSCs. Postgrafting immunosuppression consisted of PTCy (50 mg/kg/day) on days 3 and 4, followed by mycophenolate mofetil (MMF, 10 mg/kg three times daily orally or i.v.) and sirolimus (orally, monitored 2 times each week to maintain a target therapeutic plasma level of 8 to 14 ng/mL during the first 2 months after transplantation, thereafter of 5 to 8 ng/mL until discontinuation). In the absence of GVHD or disease relapse, tapering of MMF was initiated after engraftment, starting from day 20 to achieve discontinuation by day 30. Sodium 2-sulfanylethanesulfonate (mesna) and i.v. hydration were administered for uro-protection.

Stem Cell Source

Donors were mobilized by the subcutaneous administration of G-CSF for 5 to 6 days at 10 μg/kg/day. PBSCs were collected by leukapheresis to achieve a target stem cell dose of 4 to 8 × 10^6 CD34$^+$ cells per kg of patient body weight.

Supportive Care

Antimicrobial prophylaxis and treatment of infectious complications were administered per the institutional guidelines, following international recommendations [39-41]. All patients received, from the first day of conditioning, acyclovir, levofloxacin, and voriconazole. Cytomegalovirus (CMV) reactivations were monitored weekly in peripheral blood plasma samples by quantitative PCR. A pre-emptive treatment with gancyclovir or foscarnet was started when CMV DNA copy number was more than 1000 copies/mL or increased more than .5 log in peripheral blood plasma. Epstein-Barr virus (EBV) was monitored on plasma samples every 2 weeks by PCR and rituximab (375 mg/m^2) was administered as pre-emptive treatment in case of high (>1000 copies/mL) or rising DNAemia in 2 consecutive determinations; if possible, this was accompanied by reduction of immunosuppressive therapy. A regular monitoring of human herpes virus-6 (HHV-6) DNA in plasma, using a real-time PCR assay, was performed weekly within the first 100 days after HSCT, and antiviral therapy with either foscarnet or gancyclovir was started in case of possibly HHV6-related clinical manifestations.

Engraftment, Chimerism, and Relapse Detection

Neutrophil engraftment was defined as the first of 3 consecutive days with neutrophil counts ≥ .5 × 10^9/L after transplantation, and *platelet engraftment* was defined as platelet counts ≥ 20 × 10^9/L in the absence of transfusions during the preceding 7 days. Post-transplantation disease follow-up consisted of monthly BM evaluations for the first 3 months after transplantation and then 2 times each year. Additional BM evaluations were performed 4.5 and 9 months after HSCT in patients with high-risk acute leukemia. We assessed donor-recipient chimerism on unfractionated BM aspirate samples with a commercial assay based on quantitative PCR detection of insertion/deletion polymorphisms, all outside the HLA complex (AlleleSEQR Chimerism Assay, Celera Genomics, Rockville, MD). In the cases for which informative patient-specific polymorphisms were not available, short-tandem repeats analysis (AmpFISTR Profiler Plus PCR Kit; Applied Biosystem, Carlsbad, CA) was used. Patients were considered fully chimeric if their unfractionated BM samples were ≤ 5% host or ≥ 95% donor, depending on the technique used.

In case of acute leukemia *relapse*, defined as the presence of >5% of leukemic blasts in the BM aspirate, eventual genomic loss of the mismatched HLA was performed either by low-resolution typing or by allele-specific quantitative PCR, as previously described [42,43]. In case of

suspected HLA-loss relapse in the presence of low blast burden (<20%), leukemic cells were fluorescent activated cell sorter–purified and confirmatory genomic HLA typing was performed on sorted cells.

Immune Reconstitution

We studied immune reconstitution dynamics by flow cytometry. Data were analyzed with the FCS Express software (De Novo Software, Glendale, CA). Absolute cell counts were determined on the CD45bright/SSClow population with fluorochrome-conjugated monoclonal antibodies to CD3, CD4, CD8, CD16 and CD56, TCRαβ, TCRγδ, and TrueCount beads (Beckman Coulter, Brea, CA). *B cell reconstitution* was defined as B cell counts ≥ 1 cells/μL. In a group of patients, selected based on sample availability, we analyzed the frequencies of circulating $CD4^+$ recent thymic emigrants (RTEs), defined as $CD45RA^+CD62L^+CD95^-CD31^+$ and $CD4^+$ Treg, defined as $CD25^+CD127^-Foxp3^+$. Samples were acquired on a Fortessa cytometer (Becton Dickinson, Franklin Lakes, NJ) and analyzed with FlowJo software version 9.8 (Tree Star Inc., Ashland, OR).

GVHD Grading and Treatment

Clinical diagnosis and grading of acute GVHD (aGVHD) were made according to the consensus criteria [44,45]. Chronic GVHD (cGVHD) diagnosis and grading were based on the National Institutes of Health consensus criteria [46,47]. GVHD was treated per institutional protocols, considering the European Society for Blood and Marrow Transplantation-European Leukemia Net recommendations [48].

Statistical Analysis

Outcomes are reported as of November 2014. Probabilities of overall survival (OS) and disease-free survival (DFS) were estimated using the Kaplan-Meier method, and cumulative incidences (CI) were provided. CI of NRM, relapse, aGVHD, and cGVHD were computed to take into account the presence of competing risks. Specifically, in calculating the CI rates of NRM, the competing risk was relapse; when calculating relapse, the competing risk was NRM. When calculating neutrophil and platelet recovery, the competing risk was death. For aGVHD and cGVHD, the competing risks were relapse and death. All patients were considered evaluable for aGVHD analysis, and those who had a documented engraftment and a follow-up >100 days were evaluated for cGVHD. *P* values were considered significant if < .05. Statistical analyses were performed with SPSS version 13.0 and R version 2.12.2 (R Project for Statistical Computing, http://www.r-project.org/), package "cmprsk" [49]. Statistical analyses of immune reconstitution data were performed with Prism 5 (GraphPad Software, Carlsbad, CA).

RESULTS

Patients and Graft Characteristics

Patients, donor, and graft characteristics are provided in Table 1. All subjects had high-risk hematologic malignancies, with the most common diagnosis being acute myeloid leukemia (55%). A total of 25 patients (62.5%) had active disease at transplantation, whereas 15 (37.5%) were in hematologic remission. Twelve patients (30%) received a prior autologous HSCT and 9 patients (22.5%) received a prior allogeneic HSCT (4 HLA identical, 3 MUD, and 2 cord blood transplantations). Nineteen (61%) of the 31 patients who underwent haplo-PTCy as first allogeneic transplantation scored high/very high according to DRI, 12 patients (39%) scored intermediate, and no patient had a low disease score. Eleven (27.5%) patients received the transplant from siblings, 5 from parents, and 22 from sons or daughters. The majority of donor-recipient pairs (85%) were mismatched for 5 of 10 HLA loci. The median age of patients was 55 years (range, 21 to 78) and median age of donor was 36 years (range, 20 to 68).

Table 1
Patient, Donor, and Transplantation Characteristics

Characteristic	Value
No. of patients	40
Age, median (range), yr	55 (21-78)
Gender	
Male	23
Female	17
Disease	
AML	22 (CR1 = 3, CR > 1 = 3, rel/ref = 16)
ALL	5 (CR1 = 2, CR > 1 = 1, rel/ref = 2)
MDS	1 (ref = 1)
CML	1 (blast crisis = 1)
HD	6 (CR > 1 = 3, SD/PD = 3)
NHL	5 (CR1 = 1, CR > 1 = 2, PR = 1, PD = 1)
DRI	
Low	0
Intermediate	12
High	13
Very high	6
Previous allogeneic HSCT	9
Days from diagnosis to allogeneic HSCT, median (range)	461 (100-2896)
Comorbidity-age index	
0	1
1-2	10
≥3	28
Not evaluable	1
HLA mismatches	
5	34
3-4	4
1-2	2
Predicted NK alloreactivity	13
Graft composition	
$CD34^+$ cells × 10^6/kg, median (range)	6.02 (4.15-8.02)
$CD3^+$ cells × 10^8/kg, median (range)	2.22 (1.05-4.83)
CMV status (donor/recipient)	
Positive/positive	19
Negative/positive	16
Positive/negative	1
Negative/negative	4

AML indicates acute myeloid leukemia; CR, complete remission; rel/ref, relapsed/refractory; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; HD, Hodgkin's disease; SD, stable disease; PD, progressive disease; NHL, non-Hodgkin lymphoma; NK, natural killer.

Engraftment and Chimerism

PBSCs were collected by leukapheresis and infused without ex vivo manipulation. Median $CD34^+$ and $CD3^+$ cell doses were 6.02×10^6/kg (range, 4.15 to 8.02) and 2.22×10^8/kg (range, 1.05 to 4.83), respectively. The conditioning regimen induced absolute grade 4 neutropenia in all patients. Median time to neutrophil counts > $.5 \times 10^9$/L was 18 days (range, 13 to 45 days) and to platelet counts > 20×10^9/L was 16 days (range, 9 to 100 days) (Table 2). At 30 days after HSCT, neutrophil engraftment occurred in 100% of the patients and platelet engraftment in 82.5% (Figure 1A,B). At first BM evaluation, performed 30 days after HSCT, 34 of 36 evaluable patients (94%) presented full-donor chimerism, whereas the remaining 2 patients displayed hematological disease persistence. No case of primary graft failure or persistent mixed chimerism was documented. Median time of discharge after transplantation was 32 days (range, 20 to 187).

Transplantation-Related Complications and Infections

Transplantation-related complications and infections are outlined in Table 2. Sirolimus-related posterior reversible encephalopathy syndrome developed in 1 patient and resolved with sirolimus discontinuation. Severe mucositis occurred in 6 patients (15%). No case of hepatic veno-occlusive disease or HSCT-associated thrombotic microangiopathy was observed. Five patients (12%) developed severe bacterial infections: 2 gram-negative sepsis (1 carbapenemase-producing *Klebsiella pneumonia*, 1 *Stenotrophomonas maltophilia*), 1 pulmonary tuberculosis, and in

Table 2
Complications and Infections

Complications and Infections	n
Mucositis grade III	6
PRES	1
Hemorrhagic cystitis	
Mild	4
Requiring treatment	5
Severe bacterial infections	
G-sepsis	2
Tbc	1
Unknown etiology	2
Viral infections	
CMV reactivation	22
EBV reactivation	6
HHV6 positivity	25
Other	1 (enterovirus)
IFI	
Before HSCT	11 (possible = 5, probable = 6)
After HSCT	5 (possible = 1, probable = 4)

PRES indicates posterior reversible encephalopathy syndrome; G, gram-negative; Tbc, tuberculosis.

the remaining 2 cases, the causative agents were not identified. Hemorrhagic cystitis developed in 9 patients (22%), with concomitant BK virus positivity in 7 patients (78%), 1 related to JC virus and the last associated with previous radiotherapy. Five patients required treatment with bladder irrigation and/or analgesics, and cystitis resolved in all 9 patients. CMV reactivation occurred in 22 of 35 patients (63%) who were at high risk of reactivation (donor seropositive/seronegative, recipient seropositive). Six patients, all receiving transplants from seropositive donors, developed CMV disease (2 colitis, 3 pneumonia and 1 encephalitis), and the median time to CMV reactivation was 33 days (range, −9 to 100). EBV DNAemia was detected in 6 patients (15%), of whom 4 required pre-emptive treatment with rituximab. No case of EBV post-transplantation lymphoproliferative disorder was observed. One patient developed enterovirus-related hepatitis and myocarditis. Twenty-five patients (62.5%) developed HHV6 positivity (15 on peripheral blood, 7 on gut biopsies, 2 on cerebrospinal fluid, 1 on bronchoalveolar lavage) and 15 (60%) received foscarnet. Eleven patients (27%) had invasive fungal infection (IFI) before transplantation, but importantly, IFI was not a cause of mortality for any of these patients after HSCT. Of the remaining 29 patients without IFI before HSCT, 5 patients developed a possible (n = 1) or probable (n = 4) IFI after transplantation. Importantly, all infectious events occurred within the first 6 months after transplantation, before immune suppression discontinuation, which occurred at a median of 28 days after transplantation (range, 24 to 58) for MMF and a median of 201 days (range, 25 to 461) for sirolimus.

Kinetics of Immune Reconstitution

The median lymphocyte counts, performed at 30, 90, 180, and 365 days after HSCT, are depicted in Figure 2. All evaluable patients achieved rapid T cell reconstitution (Figure 3A-C), showing median cell counts at day 30 after HSCT of 338 $CD3^+$ cells/μL (range, 5 to 2564 cells/μL). The T cell repertoire was skewed toward $CD8^+$ cells, as frequently observed after allogeneic HSCT. The median time to $CD4^+ > 200/\mu L$ was 41 days (range, 21 to 107). CD31 expression on $CD4^+$ naïve T cells was analyzed to quantify RTEs. All patients had a significantly higher proportion of circulating RTEs at day 30 and 180 compared with their pre-HSCT levels, suggesting that thymic function was not impaired, and possibly even improved, after HSCT (Figure 3D). NK cell reconstitution was similarly fast (Figure 2E), with median cell counts on day 30 after HSCT of 19 $CD3^-CD56^+CD16^+$ cells per μL (range, 0 to 417 cells/mL). Median time to B cell reconstitution was 41 days (range, 24 to 278) (Figure 2F).

GVHD

The CI of grade II to IV and III and IV acute GVHD (aGVHD) at day 100 after HSCT were 15% and 7.5%, respectively (Figure 3A and Table 3). There was no significant correlation between the number of infused $CD3^+$ cells and aGVHD: the CI rates of grade II to IV aGVHD were 15% and 20% for patients receiving less or more than the median number of $CD3^+$ cells (2.22×10^8/kg), respectively ($P = .12$). Tregs have been shown to play a role in GVHD prevention by PTCy both in preclinical models and in humans [23,24]. To verify the hypothesis that the combination of PTCy and sirolimus could promote Treg expansion after transplantation, we closely evaluated Treg dynamics in our cohort of patients. Indeed, we found that circulating donor-derived Tregs were significantly expanded at day 15 after transplantation, compared with both leukapheresis samples and healthy controls (Figure 3B). Of notice, patients not experiencing aGVHD displayed significantly higher percentages of circulating Tregs at day 15 after HSCT compared with those who subsequently developed aGVHD (Figure 3C). Thirty-one patients were evaluable for cGVHD.

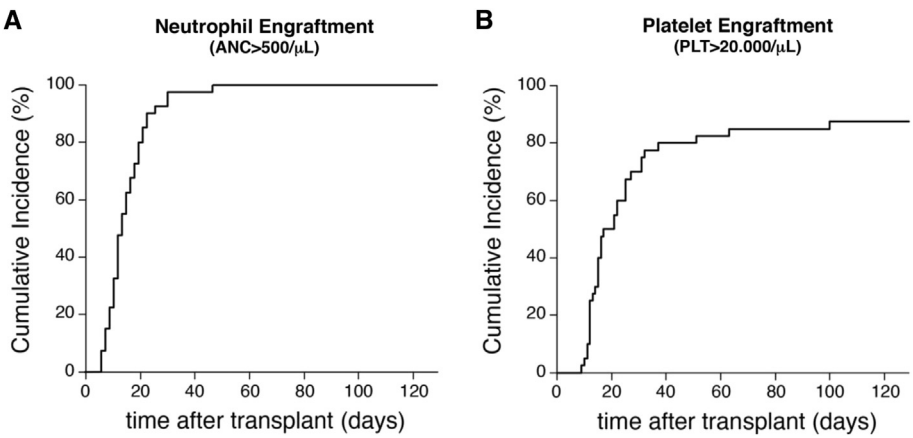

Figure 1. Cumulative incidence of (A) neutrophil engraftment and (B) platelet engraftment.

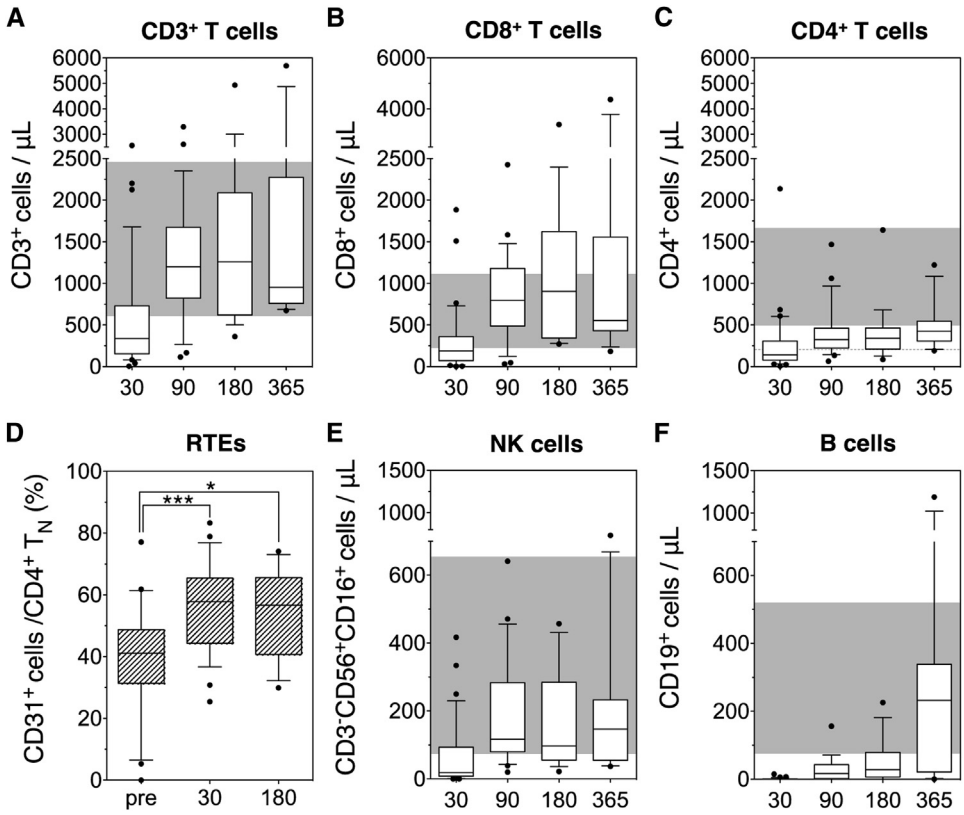

Figure 2. Immune reconstitution. Absolute counts of (A) $CD3^+$ T cells, (B) $CD8^+$ T cells, and (C) $CD4^+$ T cells. (D) Flow-cytometric quantification of circulating recent thymic emigrants (expressed as percent of $CD31^+$ cells on $CD4^+$ naïve T cells) before (pre), and after 30 and 180 days from transplantation. (E) Absolute counts of $CD56^+CD16^+CD3^-$ NK cells. (F) Absolute counts $CD19^+$ B cells circulating in peripheral blood of patients at different time points, namely 30, 90, 180, and 365 days after transplantation. *Grey shadows* denote normal reference values for each subset analyzed. * $P < .05$; *** $P < .001$.

The CI of moderate and severe cGVHD at 1 year was 20%, whereas the CI of severe cGVHD was 5.1% (Figure 3D). The median time of onset of cGVHD was 141 days from HSCT (range, 83 to 408).

NRM

The CI of NRM at day 100 and at 1 year was 12% and 17%, respectively, with most of the events occurring in the first 3 months after transplantation (median, day 63) (Figure 4). Infections were the primary cause of death in 6 patients (15%): 2 sepsis, 3 pneumonia (2 caused by CMV, 1 by *Mycobacterium tuberculosis*), and 1 hepatitis/myocarditis. One patient died from complications associated with aGVHD and concomitant CMV pneumonia. Notably, only 1 patient who underwent Sir-PTCy as second transplantation died of NRM, with the CI of NRM at 100 days and 1 year for second transplantation being 11%.

Relapse

Relapse incidence was 35% 1 year after HSCT (Figure 5A). In the subgroup of patients affected by acute leukemia, we documented 11 cases of relapse after HSCT (Figure 5B): 3 (27% of relapses) were due to immune escape leukemic variants characterized by selective genomic loss of the HLA haplotype mismatched between patient and donor [50,51]. Of note, 1 of the HLA-loss relapses was observed, for the first time to our knowledge, in a patient affected by acute lymphoblastic leukemia. In line with previous reports [10,43], HLA-loss leukemia relapses occurred at a median of 300 days after HSCT (range, 255 to 414), whereas "classical" relapses at a median of 94 days after HSCT (range, 37 to 197). Among patients with acute myeloid leukemia, no difference in relapse incidence was observed according to the presence or absence of predicted NK alloreactivity in the graft-versus-host direction ($P = .52$).

Survival and DFS

With a median follow-up of over 15 months (range, 5 to 24 months, with 25% of living patients with a follow-up $\leq$ 12 months), the estimated 1-year probabilities of OS and DFS were 56% and 48%, respectively (Figure 6A). DFS was 71% for patients who underwent transplantation in remission and 34% for those patients who underwent transplantation with active disease ($P = .01$, Figure 6B). A total of 8 patients (32%) with active disease at transplantation were alive, and 6 (75%) were alive and in remission at a median of 511 days after transplantation. There was a trend towards longer DFS in patients with more favorable DRI: 1-year DFS was 64% for patients with intermediate DRI and 41% for patients with high/very high DRI ($P = .07$) (Figure 6C). Interestingly, patients who had relapsed after a previous allogeneic HSCT displayed DFS superior to that of patients with high/very high DRI (DFS at 1 year 42%).

DISCUSSION

In the present report, we present our investigational experience on the combination of myeloablative conditioning, PBSC as stem cell source, and a CNI-free, sirolimus-based GVHD prophylaxis in the context of haploidentical HSCT with PTCy. Our group demonstrated previously that a sirolimus-

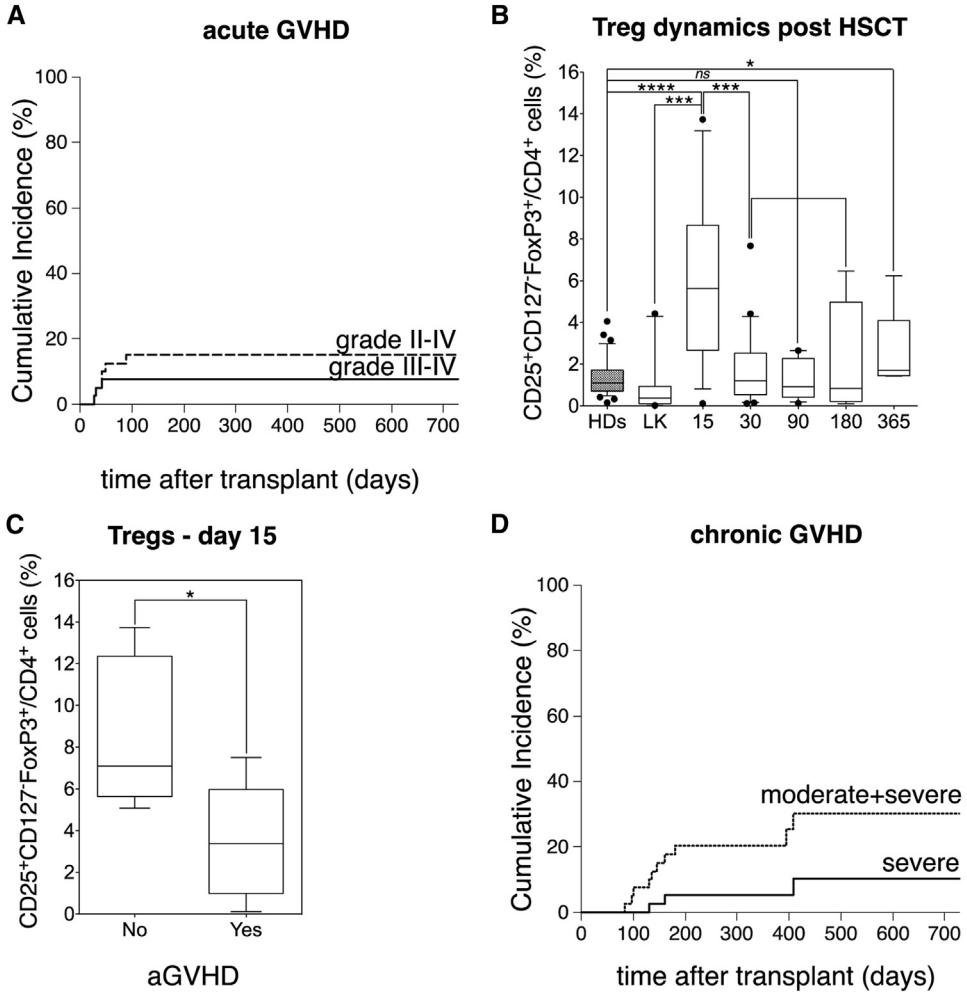

Figure 3. GVHD. (A) Cumulative incidence of grade II to IV (*dashed line*) and grade III and IV (*full line*) aGVHD. (B) Frequency of regulatory T cells (defined as $CD25^+CD127^-FoxP3^+$ $CD4^+$ T cells) circulating in the peripheral blood of patients at different time-points after transplant and compared with the frequency in both healthy donors' peripheral blood (HDs) and the leukapheresis product (LK) infused. (C) Comparison of Treg frequencies at day 15 in patients subsequently developing or not aGVHD. (D) Cumulative incidence of overall (*dashed line*) and severe cGVHD (*full line*) classified according to NIH criteria. * $P < .05$; *** $P < .01$; **** $P < .001$. ns indicates not significant.

based GVHD prophylaxis enables T cell–replete PBSC transplantation from haploidentical donors, but such approach was limited by a relatively high NRM. Building on our previous experience, here we incorporated PTCy in the context of a sirolimus-based GVHD prophylaxis. We hypothesized that sirolimus and PTCy could synergize providing adequate GVHD protection without excessively increasing the risk of transplantation-related morbidities, especially in the setting of patients with active disease and/or high DRI at transplantation. We show that our modified haplo-PTCy platform allows for a high rate of engraftment, with acceptable NRM and GVHD rates, and encouraging remission rates. Importantly, despite the advanced age of the patients studied (median age of 51 years, with 25% of patients older than 65), the comorbidities (approximately two thirds of patients had a comorbidity-age score of 3 or higher), and the relative poor risk status of their malignancies (with the majority of patients having a high or very high DRI), NRM was 12% at 100 days and 17% at 1 year. This is in the range of NRM reported for other PTCy-based approaches [15-17,52]. Thus, our results indicate that intensification of the conditioning regimen, coupled with the use of PBSC, is feasible and does not

Table 3
Clinical Outcomes

Outcome	Value
Follow-up of surviving patients, median (range), d	451 (140-728)
Engraftment	
Time to neutrophil .5 × 10^9/L, median (range), d	18 (13-45)
Time to platelets 20 × 10^9/L, median (range), d	16 (9-100)
aGVHD, n	
Grade II-IV	7
Grade III-IV	3
cGVHD, n	
No	19
Minimal	2
Moderate	7
Severe	3
Day +100 NRM, n	5
Overall NRM, n	7
Relapse, n	
Overall	13
AL relapses	11/27
Classical	8/11
HLA-loss	3/11
Relapse-related death, n	10
Surviving, n	23

NRM indicates non-relapse mortality; AL, acute leukemia.

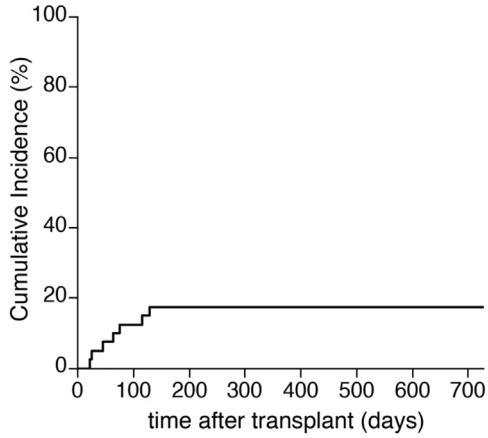

Figure 4. Cumulative incidence of NRM.

adversely affect outcomes in the haplo-PTCy context, as also recently reported [18,19,53].

An unexpected finding of this study was a high incidence of viral reactivations. Although the overall mortality associated with these complications was limited, as evidenced by the favorable NRM, it caused significant morbidity in patients and often required aggressive antiviral therapy. Similar observations have been reported by 2 other independent groups [19,54]. Such a high incidence of viral infections, combined with the relatively low incidence of severe bacterial and mold infections, might depend, at least in part, on PTCy's mechanism of action. Preclinical studies have shown that cyclophosphamide administered early after HSCT preferentially kills activated, cycling alloreactive, predominantly memory [55] T cells while sparing resting, nonalloreactive T cells [56]. Given the high incidence of viral events, we might speculate that alloreactivity, at least in the setting of PBSC grafts, largely overlaps with cross reactivity of antiviral T cells [57]. However, despite the high incidence of viral reactivations, the overall immune reconstitution profile was favorable, as evidenced by a median time to $CD4^+ > 200/\mu L$ of only 41 days. This immune reconstitution kinetics was faster compared with previous haplo-PTCy reports [52] and could be related to the use of PBSC instead of BM grafts.

The incidences of acute and chronic GVHD were low and similar to those reported previously for haplo-PTCy using BM. Notably, the 15% CI of grade II to IV aGVHD we observed is lower than those of other haplo-PTCy studies using PBSC as graft source, which range from 30% to 53% at 1 year [15-17,19]. In our setting, the low GVHD incidence might be, at least in part, explained by a significant early increase in circulating regulatory T cells at day 15 after HSCT, possibly as an effect of the combined activity of PTCy and sirolimus in the absence of CNIs. Both agents have been reported to either favor in vivo Treg expansion [10,25] or to selectively spare this T cell subset [23,24]. Patients with active GVHD display lower Treg frequencies compared with those without GVHD [58,59]. Accordingly, we found that values of circulating Tregs $< 5\%$ at day 15 after transplantation were predictive of subsequent GVHD occurrence. Overall, these observations suggest that replacement of standard CNI-based GVHD prophylaxis with sirolimus helps prevent GVHD by a synergic action with PTCy on the Treg pool early after transplantation.

Nevertheless, a crucial problem, especially in high-risk patients, is relapse of the original disease. This has been particularly relevant with PTCy, given the significant rate of disease relapse observed. Although in our study overall efficacy is difficult to assess given the small numbers, the limited follow-up, and the poor disease risk of many patients, survival seems promising. The 1-year OS of 56% and DFS of 48% are encouraging considering that approximately one fourth of our patients had a prior allogeneic HSCT and one half underwent transplantation with active disease. Of the 25 patients who underwent transplantation in the presence of active disease, the current DFS is 34%, which is nonetheless comparable with the other published haplo-PTCy reports. Yet, for patients who undergo transplantation with chemoresistant disease, relapse rates remains critical, despite intensification of the conditioning and the use of PBSC. In this respect, an important concern about PTCy has been that effective purging of alloreactive T cells might be the cause of dampened graft-versus-tumor effect. Two observations strongly argue against this hypothesis. First, we report on several patients affected by chemoresistant disease, high or very high DRI score, or who had relapsed after a previous allogeneic HSCT, achieving sustained remissions. Secondly, a strong indicator of an antileukemic effect mediated by donor-derived T cells (despite effective PTCy-mediated

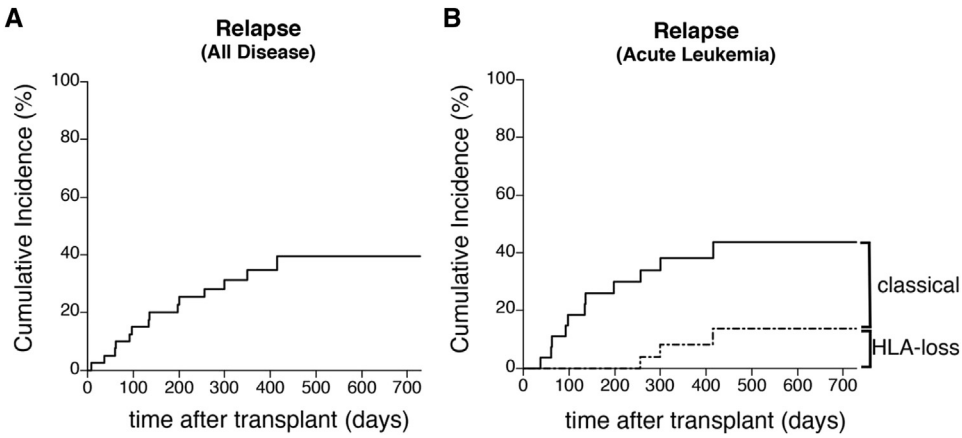

Figure 5. Disease relapse. (A) Cumulative incidence of disease relapse in the entire patient cohort. (B) Disease relapse in patients with acute leukemia; the *dotted curve* represents cumulative incidence of relapses due to genomic loss of the mismatched HLA in leukemic cells (HLA-loss variants), over which are cumulated classical bone marrow relapses (full line).

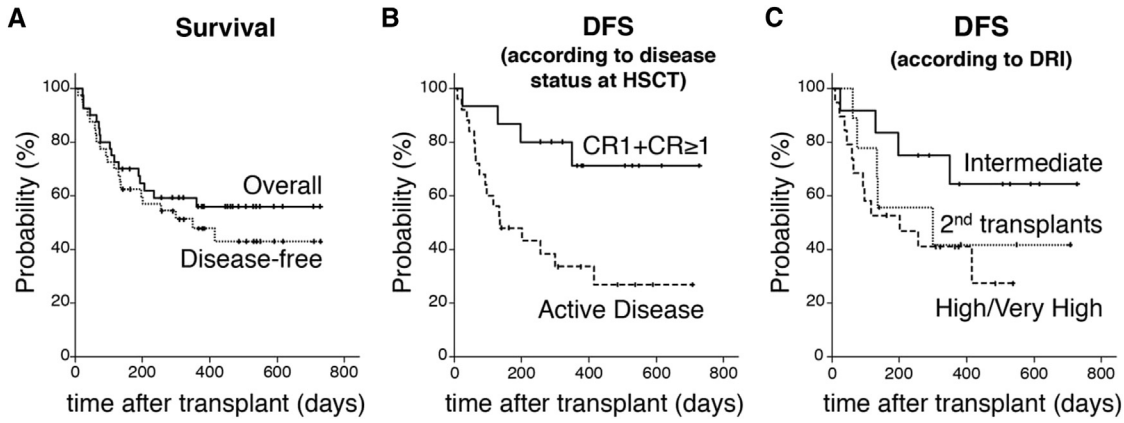

Figure 6. Survival. (A) Kaplan-Meier estimates of disease-free (*dashed line*) and overall (*full line*) survival in the entire patient cohort. (B) DFS stratified according to the disease status at transplantation. (C) DFS stratified according to the disease risk index.

taming of alloreactivity) is the presence of leukemic relapses characterized by genomic loss of mismatched HLA, a mechanism we previously characterized for acute myeloid leukemia [43,50,51], potentially favoring leukemia immune evasion. Of note, here we have extended the spectrum of hematological malignancies exploiting this mechanism by reporting for the first time an HLA-loss relapse in a patient affected by acute lymphoblastic leukemia. Thus, the observation of HLA-loss relapses across different diseases suggests that this mechanism of immune evasion might be considered as a surrogate marker of post-transplantation alloreactivity directed against hematological malignancies.

Notably, 9 patients affected by acute leukemia included in this study underwent Sir-PTCy haploidentical transplantation as their second allogeneic HSCT (HSCT2). The choice of donor for HSCT2 is controversial, and few studies have evaluated the effect of donor change on transplantation outcomes [60-63]. Indeed, in our cohort, 42% of patients receiving haploidentical HSCT2 for leukemia relapse after allogeneic transplantation could be rescued, with low rates of NRM. Thus, although the patient number is too small to draw a definite conclusion, our data suggest that haploidentical HSCT using the Sir-PTCy platform is safe and feasible for patients with acute leukemia who relapse after a first allogeneic HSCT.

In conclusion, our study, although limited by a small number of patients with relatively short follow-up, suggests that G-CSF–mobilized PBSC is a viable alternative to BM as a graft source for haplo-PTCy in high-risk diseases, and that sirolimus-based GVHD prophylaxis allows valid GVHD prevention in this context. The low rates of GVHD and NRM, as well as the favorable immune reconstitution profile, of our Sir-PTCy platform pave the way for a prospective comparative trial comparing BM and PBSC in this specific transplantation setting.

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